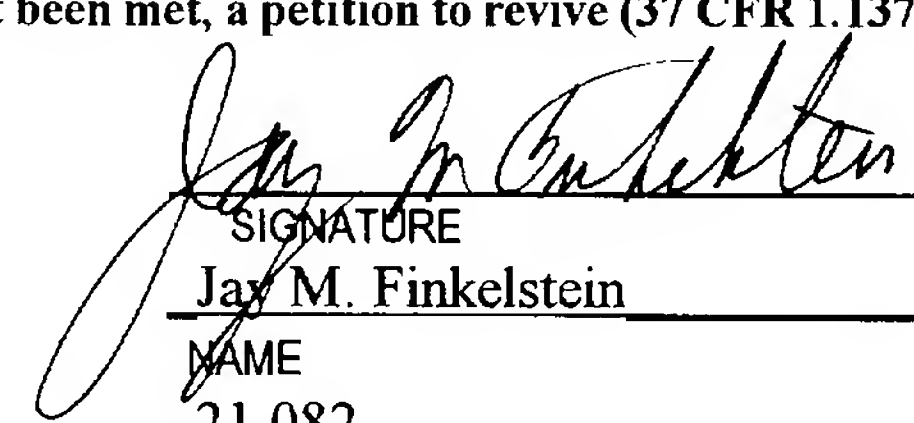


JG18 Rec'd PCT/TO 0 4 MAR 2002

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>MAYUMI=1</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (If known, see 37 CFR 1.5)  <b>10/070252</b>
INTERNATIONAL APPLICATION NO. <b>PCT/JP00/01267</b>	INTERNATIONAL FILING DATE <b>03 March 2000</b>	PRIORITY CLAIMED <b>03 September 1999</b>
TITLE OF INVENTION <b>METHOD OF INTRACELLULAR SUSTAINED-RELEASE OF DRUG AND PREPARATIONS</b>		
APPLICANT(S) FOR DO/EO/US <b>Tadanori MAYUMI et al.</b>		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li><input type="checkbox"/> is attached hereto (required only if not transmitted by the International Bureau).</li> <li><input checked="" type="checkbox"/> has been communicated by the International Bureau.</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been communicated by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li><input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p><b>Items 11. to 16. below concern document(s) or information included:</b></p> <ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li><input type="checkbox"/> An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input type="checkbox"/> A FIRST preliminary amendment.</li> <li><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input checked="" type="checkbox"/> Other items or information:             <ul style="list-style-type: none"> <li><input checked="" type="checkbox"/> Courtesy copy of the International Application as filed (In Japanese).</li> <li><input checked="" type="checkbox"/> Courtesy copy of the first page of the International Publication (WO 01/17511).</li> <li><input checked="" type="checkbox"/> Courtesy copy of the International Preliminary Examination Report. There were no annexes.</li> <li><input checked="" type="checkbox"/> Formal drawings, 2 sheets, Figures 1-4.</li> <li><input checked="" type="checkbox"/> Courtesy Copy of the International Search Report.</li> <li><input checked="" type="checkbox"/> Application Data Sheet</li> </ul> </li> </ol> <p><input checked="" type="checkbox"/> The application is (or will be) assigned to: CHUGAI SEIYAKU KABUSHIKI KAISHA, whose address is 5-1, Ukima 5-chome, Kita-ku., Tokyo 115-8543 Japan.</p>		

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>10/070252</b>		International Application No. <b>PCT/JP00/01267</b>	Attorney's Docket No. <b>MAYUMI=1</b>
<b>17. [xx]</b> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... <b>\$1040.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... <b>\$890.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... <b>\$740.00</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)–(4)..... <b>\$710.00</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)–(4)..... <b>\$100.00</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>			<b>CALCULATIONS PTO USE ONLY</b>
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			<b>\$ 130.00</b>
Claims as Originally Presented	Number Filed	Number Extra	Rate
Total Claims	- 20		X \$18.00
Independent Claims	- 3		X \$84.00
Multiple Dependent Claims (if applicable)			+\$280.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>			<b>\$1,020.00</b>
Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate
Total Claims	- 20		X \$18.00
Independent Claims	- 3		X \$84.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>			<b>\$1,020.00</b>
Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.			<b>\$</b>
<b>SUBTOTAL =</b>			<b>\$1,020.00</b>
Processing fee of <b>\$130.00</b> for furnishing the English translation later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			<b>\$1,150.00</b>
<b>TOTAL NATIONAL FEE =</b>			<b>\$1,150.00</b>
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +			<b>\$</b>
<b>TOTAL FEES ENCLOSED =</b>			<b>\$1,150.00</b>
			<b>Amount to be:</b>
			<b>refunded</b>
			<b>charged</b>
a. [ ] A check in the amount of \$ _____ to cover the above fees is enclosed.			
b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of \$ 1,150.00, is attached.			
c. [ ] Please charge my Deposit Account No. <b>02-4035</b> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.			
d. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <b>02-4035</b> . A duplicate copy of this sheet is enclosed.			
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>			
SEND ALL CORRESPONDENCE TO:			
<b>BROWDY AND NEIMARK, P.L.L.C.</b> <b>624 NINTH STREET, N.W., SUITE 300</b> <b>WASHINGTON, D.C. 20001</b> <b>TEL: (202) 628-5197</b> <b>FAX: (202) 737-3528</b> <b>Date of this submission: March 4, 2002</b>			
			 SIGNATURE <b>Jay M. Finkelstein</b> NAME <b>21,082</b> REGISTRATION NUMBER

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JC13 Rec'd PCT/PTO 04 MAR 2002

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## APPLICATION INFORMATION

Title Line One:: METHOD AND FORMULATION FOR SUSTAINED INT  
Title Line Two:: RACELLULAR DRUG RELEASE  
Total Drawing Sheets:: 2  
Formal Drawings?:: Yes  
Docket Number:: MAYUMI=1  
Secrecy Order in Parent Appl.?:: No

## REPRESENTATIVE INFORMATION

Representative Customer Number:: 1444

CONTINUITY INFORMATION

This application is a:: 371 OF  
> Application One:: PCT/JP00/01267  
Filing Date:: 03-03-2000

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: 249845/1999  
Filing Date:: 09-03-1999  
Country:: Japan  
Priority Claimed:: Yes

Source:: PrintEFS Version 1.0.1

10070252 1070802

10/070252 #3

Rec'd PCT/PTO 08 JUL 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: MAYUMI=1

In re Application of:	)	Art Unit:
	)	
Tadanori MAYUMI et al	)	Examiner:
	)	
I.A.: PCT/JP00/01267	)	Washington, D.C.
I.A. Date: March 3, 2000	)	
	)	Confirmation No. 9722
US Appln. No.: 10/070,252	)	
Nationalized: March 4, 2002	)	
	)	
For: METHOD AND FORMULATION FOR	)	July 8, 2002
SUSTAINED INTRACELLULAR...	)	

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified  
application, please amend as follows:

IN THE CLAIMS

Attached hereto is a marked-up version of the  
changes made to the claims by the current amendment. The  
attached version is captioned "Version with Markings to Show  
Changes Made".

Amend claims 3, 4 and 6 as follows:

In re of Appln. No. 10/070,252

• • •

3. (amended) The composition according to claim 1 ,  
wherein the fusogenic capability derives from Sendai virus.

4. (amended) The composition according to claim 1,  
wherein the physiologically active substance is selected from  
the group consisting of low-molecular weight drugs, proteins  
and genes.

6. (amended) A method of introducing a  
physiologically active substance into an animal by contacting  
a viable cell with the composition according to claim 1.

Add the following new claims:

7. (new) The composition according to claim 2,  
wherein the fusogenic capability derives from Sendai virus.

8. (new) The composition according to claim 2,  
wherein the physiologically active substance is selected from  
the group consisting of low-molecular weight drugs, proteins  
and genes.

9. (new) A method of introducing a physiologically  
active substance into an animal by contacting a viable cell  
with the composition according to claim 2.

In re of Appln. No. 10/070,252

• • • • •

10. (new) A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to claim 3.

11. (new) A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to claim 4.

12. (new) A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to claim 7.

13. (new) A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to claim 8.

# REMARKS

The above amendments of claims 4 and 6 are made to eliminate improper multi dependency, i.e. in order to make such claim consistent with 37 CFR 1.75(c) so that all claims will be examined. Claim 3 is amended to eliminate the remaining multidependent claim in order to reduce filing fees. New claims 7-10 are submitted herewith to reinsert in part the claim variation of the original claims. The amendments are clearly not "narrowing" amendments. The scope of the claims



In re of Appln. No. 10/070,252

...


has not been changed; no limitations have been added and none are intended.

Applicants respectfully await the results of a first examination on the merits.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
Attorneys for Applicants

By

  
Sheridan Neimark  
Registration No. 20,520

SN:jaa

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In re of Appln. No. 10/070,252

Version with Markings to Show Changes Made

3. (amended) The composition according to claim 1 ~~or 2~~, wherein the fusogenic capability derives from Sendai virus.

4. (amended) The composition according to ~~any one of claims 1-3~~claim 1, wherein the physiologically active substance is selected from the group consisting of low-molecular weight drugs, proteins and genes.

6. (amended) A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to ~~any one of claims 1-4~~claim 1.

SPECIFICATION

METHOD AND FORMULATION FOR  
SUSTAINED INTRACELLULAR DRUG RELEASE

(Technical Field)

5           This invention relates to the technology of  
introducing drugs, proteins, genes and other  
physiologically active substances directly into cells and  
then allowing the introduced physiologically active  
substances to be released slowly in the cells. More  
10 particularly, the invention relates to a composition  
containing a physiologically active substance of interest  
which can be introduced directly into a cell and which  
thereafter can be released slowly in the cell. The  
invention also relates to a method for allowing the  
15 physiologically active substance of interest to be directly  
introduced cells and then released slowly in the cells.

(Background Art)

For successful gene therapy and treatment with novel  
vaccines, efforts are being made today in order to ensure  
20 that biopolymers such as genes and antigenic proteins are  
introduced into the cytoplasm directly and efficiently.  
Unlike low-molecular weight drugs, high-molecular weight  
substances such as genes, antigenic proteins and  
physiologically active proteins are not only low in  
25 membrane permeability, absorption and tissue migration but  
they also undergo rapid degradation in blood. It has been  
desired to develop a technology by which those polymeric,  
physiologically active substances can be introduced into

the cytoplasm in a desired and efficient manner without causing damage to the cell.

Liposomes can hold a number of substances and are still biocompatible, so they have drawn researchers' attention as carriers for transporting physiologically active substances. However, with liposomes, the efficiency of introducing the desired substance into a cell is so low that it can hardly be introduced into the cytoplasm. To deal with this difficulty, various proposals have been made, including modifying the surfaces of liposomes with lectins, antibodies, etc. so that they are positively bound to cell surfaces. In fact, however, liposomes, whether surface-modified or not, are taken up by cells via endocytosis, so they are lysed by an enzyme called lysozyme and the proportion in which the substance of interest is actually transferred into the cytoplasm is at an extremely low level.

It was reported that in order to overcome this difficulty, fusogenic liposomes having the fusogenic capability of Sendai virus were developed as carriers that could be directly introduced into the cytoplasm via the cell membrane. Such fusogenic liposomes can be prepared by forming a complex between a liposome and the coat protein of Sendai virus which mediates fusion to the cell membrane. The prepared fusogenic liposomes have an almost comparable fusogenicity to Sendai virus and it has been reported that by encapsulating genes, proteins or other high-molecular weight substances, the substance of interest can be

directly introduced into the cytoplasm with high efficiency but without causing cell injury ["DDS", Journal of the Japan Society of DDS, Vol. 13, No. 1, January 1998, pp. 21-26 and 27-33].

5 Even if the fusogenic liposomes are used as carriers for physiologically active substances, the release of the physiologically active substance in cells cannot be controlled, so the substance introduced into the cell is released at a time and its activity (toxicity) is not  
10 sustained. Take, for example, the case of introducing a gene into the cytoplasm; the gene is decomposed in the cytoplasm and its expression is not sustained. In the case of a protein having pharmacological activity, the activity is not sustained and it has to be administered by an  
15 increased number of times in a larger dose. Therefore, if physiologically active substances are introduced into cells directly and efficiently and if their release is controlled within the cytoplasm, the intended physiological activity can be exhibited efficiently in the cell. It is desired to  
20 develop a transport carrier that permits a substance of interest to be introduced into cells and which enables slow release of the substance in the cell.

Desired is the development of a safe and stable transport carrier that allows physiologically active  
25 substances, in particular high-molecular weight substances such as proteins and genes, to be introduced into cells efficiently and without damaging the cell membrane and which still can control and adjust the release profile of

the introduced substance within the cell.

(Disclosure of the Invention)

The present inventors paid particular attention to nanospheres currently developed as a technique for  
5 controlled release of drugs and accomplished the present invention by combining nanospheres with fusogenic liposomes.

Active efforts have heretofore been made in an attempt to achieve controlled *in vivo* release of drugs and  
10 this has spawned a number of techniques including the nanosphere technology. Nanospheres as contemplated in the invention are vesicles that are typically made of polymeric matrices and which encapsulate a large volume of drugs or high-molecular weight substances. The rate for the release  
15 of the trapped drug can be controlled by the appropriate choice for parameters including the size of the nanospheres, the type of the matrix-forming polymer, and the degree of crosslinking within or between molecules of the polymer. However, given alone, nanospheres are not  
20 easily taken up by cells and even if they are, the uptake is by endocytosis and the efficiency of introduction is very low because of lysis by the enzyme called lysozyme.

The present inventors assumed that by combining fusogenic liposomes with nanospheres encapsulating a  
25 physiologically active substance of interest, it would be possible to achieve efficient introduction of the nanospheres into the cytoplasm while controlling the release of the encapsulated physiologically active

substance within the cytoplasm. Based on this assumption, the inventors continued their studies and finally accomplished the present invention.

The present invention is characterized in that  
 5 nanospheres encapsulating drugs, proteins, genes or other physiologically active substances are in turn encapsulated in liposomes to which is conferred the fusogenic capability of Sendai virus. Thus, the invention provides a method by which the physiologically active substances are not only  
 10 introduced into cells directly and efficiently but are also released slowly in the cytoplasm. The invention also provides a composition to be used to implement the method.  
 (Brief Description of the Drawings)

Fig. 1 is a graph showing the time profile of the  
 15 release of FITC-dextran from the poly(lactic acid) nanospheres prepared in Example 2;

Fig. 2 is an agarose gel electrophoretogram showing how encapsulated plasmid DNA having the T7 promoter sequence remained stable in the gelatin nanospheres  
 20 prepared in Example 3 as they were reacted with a rat liver homogenate;

Fig. 3 is a graph showing the relationship between the degree of crosslinking of gelatin and the release of encapsulated plasmid DNA from the gelatin nanospheres  
 25 prepared in Example 3; and

Fig. 4 is a graph showing the time profile of the intracellular expression of plasmid DNA having a T7 promoter that was encapsulated in gelatin nanospheres which

in turn were encapsulated in the fusogenic liposomes of the invention.

(Modes for Carrying Out the Invention)

The nanospheres to be used in the invention may be  
5 prepared from any conventionally known materials by any  
conventionally known methods as long as they fit the  
purpose of the invention. See, for example, Leelarasamee  
N. et al., J. Microencapsul. 5; 147-57, 1988; Singh M. et  
al., Pharm. Res. 12; 1796-1800, 1995; Rutledge L. C. et  
10 al., J. Am. Mosq. Control. Assoc. 12; 39-44, 1996; Li J. K.  
et al., J. Pharm. Sci. 86; 891-895, 1997; and Kofler N. et  
al., Int. Arch. Allergy. Immunol. 113; 424-431, 1997. The  
nanospheres to be used in the invention have particle sizes  
of 10 nm - 100 nm, preferably 20 nm - 600 nm.

15 According to the invention, a desired physiologically  
active substance is encapsulated in nanospheres which, in  
turn, are encapsulated in fusogenic liposomes to prepare a  
composition which allows the physiologically active  
substance of interest to be introduced into cells and then  
20 released slowly in the cytoplasm. Liposomes can be used  
without any particular limitations as long as they can hold  
nanospheres and they can be prepared by conventional  
methods including reverse phase evaporation [Szoka, F. et  
al., Biochim. Biophys. Acta, Vol. 601 559 (1980)],  
25 injection of ether [Deamer, D. W., Ann. N. Y. Acad. Sci.,  
Vol. 308 250 (1978)] or use of a surfactant [Bruner, J. et  
al., Biochim. Biophys. Acta, Vol. 455 322 (1976)].

Lipids to form liposome structures may be



conventional ones including phospholipids, cholesterol  
and nitrogen lipids, and phospholipids are generally  
preferred. Exemplary phospholipids include various natural  
phospholipids and their hydrogenation products, as well as  
5 synthetic phospholipids. These phospholipids may be used  
either alone or in admixture. If desired, cholesterol,  
stearylamine, alpha-tocopherol and other known additives  
for liposome formation may also be added during liposome  
formation.

10 Structurally, liposomes may be giant unilamellar  
vesicles (GUVs), large unilamellar vesicles (LUVs),  
multilamellar vesicles (MLVs) and small unilamellar  
vesicles (SUVs). The particle size of liposomes is varied  
as  $\geq 1000$  nm for GUV, 100 nm - 1000 nm for LUV, 200 - 5000  
15 nm for MLV, and  $\leq 100$  nm for SUV. For the purpose of the  
invention, the particle size is preferably about 10 nm - 10  
 $\mu$ m, more preferably 10 - 1000 nm.

Suitable lipids or mixed lipids as liposome formers,  
together with cholesterol as liposome forming additives,  
20 are dissolved in organic solvents such as tetrahydrofuran,  
chloroform and ethanol; after the mixture is put into a  
suitable vessel, the solvent is distilled off at reduced  
pressure to give a film of the liposome former on the inner  
surface of the vessel. In a typical case, a rotary  
25 evaporator is used to make a film of the lipid on the inner  
surface of a centrifugal tube. To the lipid film, a  
solution of nanospheres is added as an internal aqueous  
phase and mixed. The resulting liposome solution is

reacted with a fusogenesis promoter such as Sendai virus,  
 inactivated Sendai virus or a fusogenesis promoting protein  
 to prepare fusogenic liposomes. Sendai virus is inherently  
 nonpathogenic to humans but it is preferably illuminated  
 5 with uv light so that the viral RNA is fragmented to ensure  
 utmost safety. The fusogenic liposomes of the invention  
 are safe particles since they differ from the starting  
 liposomes only in that they have been conferred the  
 fusogenic capability of Sendai virus. The fusogenic  
 10 liposomes of the invention may typically be formed by the  
 method of Bangham et al. (Bangham A. D., J. Mol. Biol.,  
 13:238-252, 1965).

Examples of the physiologically active substance  
 that can be encapsulated in the fusogenic liposomes of  
 15 the invention include: various drugs that develop  
 physiological actions when they are introduced into  
 cells; physiologically active proteins such as hormones,  
 lymphokines and enzymes; antigenic proteins that work as  
 vaccines; genes such as those which are expressed in cells,  
 20 plasmids, and genes that are involved in the expressing of  
 specified genes that induce expression; as well as various  
 genes and antisenses that are introduced for gene therapy.  
 The technology of the invention is suitably applied to  
 physiologically active substances of high molecular weight  
 25 such as proteins and genes but they can also be applied to  
 various low-molecular weight drugs to give preferred  
 results.

Safety is not the only feature of the fusogenic

liposomes of the invention; they are also highly stable and are easy to handle. According to the method of the invention, a desired substance can be efficiently introduced into the cytoplasm without causing injury to the functions of the cell membrane which is the barrier to the ingress of substances into the cytoplasm. By the appropriate choice for the characteristics of nanospheres such as the starting materials, particle size and the degree of crosslinking, the profile of the release of the introduced active substance in the cytoplasm can be controlled and, in particular, its slow release can be realized to attain the principal object of the invention. In addition, physiologically active substances can be introduced into animals by contacting viable cells with the fusogenic liposomes of the invention.

The following reference example and working examples are provided for further illustrating the present invention but are in no way to be taken as limiting.

[Reference Example]

#### 20 Materials in Experiment

Reagents, L- $\alpha$ -dimyristoylphosphatidic acid, egg yoke phosphatidylcholine (PC) and chloroform, were purchased from NOF CORP.; cholesterol, sucrose, p-phenylene diamine and calcium ionophore were purchased from Wako Pure Chemical Industries, Ltd.; carboxy-modified 0.02- $\mu$ m yellow green fluorescent fluospheres, carboxy-modified 0.02-nm red fluorescent fluospheres (nanospheres) and calcium green-1 acetoxymethyl derivative were purchased from Molecular

Probes, Inc.; and Eagle's MEM was purchased from Nissui Pharmaceutical Co., Ltd.

#### Methods of Experiment

<Preparation and purification of liposomes and fusogenic  
5 liposomes>

Liposomes were prepared by a partial modification of the method of Bangham et al., supra. Mixed lipids (12.72 mg) [PA:PC:Chol = 1:4:5 (molar ratio)] were suspended in chloroform and a lipid film was prepared on the inner  
10 surface of a centrifugal tube using a rotary evaporator. To the lipid film, 300  $\mu$ L of a nanosphere/BBS (-) solution was added as an internal aqueous phase and the two phases were mixed by vortexing to prepare liposomes. By shaking at 37°C for 2 hours, the solution of the liposomes was  
15 reacted with Sendai virus to prepare fusogenic liposomes. To purify the fusogenic liposomes, the reaction solution was layered onto a 6%, 20%, 30%, 40% and 50% sucrose density gradient and centrifuged at 24000 rpm and 4°C for 2 h ours (SW28.1, Beckman). After centrifugation, the  
20 fusogenic liposome fractions at the 20% - 30% and 30% - 40% sucrose interfaces were recovered and washed with BSS(-) [20000 rpm, 4°C x 40 minutes (SW28.1, Beckman)]. Before use in the experiment, the fusogenic liposomes were illuminated with uv light (2000 J/m<sup>3</sup>) in order to fragment  
25 the RNA of Sendai virus.

<R18 labelling of liposomes and fusogenic liposomes>

A portion (0.1 mM) of R18/ethanol solution was added in a 1/100 volume to the liposomes or fusogenic liposomes

that were conditioned to have the same fluorescence intensity of 150000 (Ex, 490 nm; Em, 515 nm) and reaction was performed at room temperature for 1 hour. The unreacted R18 was removed by centrifuging at 25000 rpm and  
5 4°C for 40 minutes (SW55, Beckman).

<Cultured cells>

Mouse fibroblast cells Ltk<sup>-</sup> were cultured in an Eagle's MEM medium containing 10% fetal calf serum (FCS).

<Introducing nanospheres into cultured cells>

10 4 x 10<sup>4</sup> Ltk<sup>-</sup> cells were seeded on a 4-well chamber slide. After one day, the cells were washed with PBS(-) and exposed for 1 minute to the action of the nanospheres, liposomes or fusogenic liposomes (fluorescence intensity, 100000; Ex, 490 nm; Em, 515 nm). After washing with  
15 PBS(-), 0.1% p-phenylenediamine/Eagle's MEM medium was added as an anti-stain agent.

<Examination by confocal laser microscopy>

Examination was made with a confocal laser scanning fluorescence microscope, MRC-1024 (BIO-RAD). After  
20 excitation with a Kr/Ar laser, the nanospheres (yellow green fluorescent) were examined at Ex of 488 nm and Em of 540 nm (540 DF) and R18 was examined at Ex of 568 nm and Em of 585 nm (585 LP).

<Measuring the efficiency of introduction into cells with  
25 the aid of Ca<sup>2+</sup> indicator>

To the nanospheres (red fluorescent), calcium green-1 acetoxymethyl derivative was added as a fluorescent Ca<sup>2+</sup> indicator to give a concentration of 20 mM and mixed at

room temperature for 1 hour so that it was adsorbed on the nanospheres. The unadsorbed calcium green-1 was removed by dialysis (Spectra/Por Membranes MWCO:12-14000) and buffer replacement was performed to prepare liposomes and fusogenic liposomes. 2 x 10<sup>5</sup> Ltk<sup>-</sup> cells that had been seeded on 6-well plates one day before were washed with PBS(-) and exposed for 1 minute to the action of the calcium green-1 acetoxymethyl derivative, calcium green-1 adsorbed nanospheres, liposomes or fusogenic liposomes which were all conditioned to have the same fluorescence intensity of 2700 (Ex, 488 nm; Em, 534 nm). Thereafter, the cells were washed with PBS(-), cultured in an Eagle's MEM medium for 1 hour, stripped from the medium with trypsin and subjected to the measurement of fluorescence intensity (Ex, 488 nm; Em, 534 nm). Thereafter, a calcium ionophore/DMSO solution was added to give a concentration of 0.1 mg/mL and fluorescence intensity was measured at Ex = 488 nm and Em = 534 nm.

### Results and Discussion

To begin with, the fluorescence labelled nanospheres having a diameter of 20 nm were examined with a confocal laser microscope at a magnification of 10000 in order to see whether they could be encapsulated in the liposomes or fusogenic liposomes. As a result, aggregated images were obtained but the fluorescence of the 20-nm<sup>φ</sup> nanospheres was observed only in the positions on transmission micrographs where the liposomes or fusogenic liposomes were observed. Thus, it became clear that the nanospheres could be

encapsulated into liposomes and that using such liposomes, one could prepare fusogenic liposomes having the nanospheres encapsulated therein. It was confirmed that several nanospheres had been encapsulated in each liposome  
5 or fusogenic liposome.

The thus prepared fusogenic and non-fusogenic liposomes having the nanospheres encapsulated therein were labelled with R18 and the efficiency of nanosphere introduction into Ltk<sup>-</sup> cells was determined. Fluorescence  
10 was hardly detectable in the cells on which the non-fusogenic, nanosphere encapsulating liposomes or the nanospheres alone were acted upon; on the other hand, a large number of nanospheres were introduced into the cells on which the fusogenic liposomes had been acted. Unlike  
15 the nanospheres, the distribution of the red fluorescence from R18 used as the marker of the fusogenic liposomes was not observed within the cells but observed on the cell membrane. Section images of the Ltk<sup>-</sup> cells on which the fusogenic liposomes had been acted were successively taken  
20 in thicknesses of 4  $\mu$ m as calculated from the surface of attachment; nanospheres were clearly visible inside the cell membrane in conformity with the cell shape, indicating the presence of R18 fluorescence on the cell membrane. Thus, the fusogenic capability of the fusogenic liposomes  
25 was reconfirmed and it was suggested that even the nanospheres in suspension could be efficiently introduced into the cytoplasm by using the fusogenic liposomes.

These phenomena were further studied using a calcium



green-1 acetoxymethyl derivative as a  $\text{Ca}^{2+}$  indicator. The calcium green-1 acetoxymethyl derivative does not emit fluorescence on its own but when in a cell, it is hydrolyzed by an endogenous esterase to form a chelate with  $\text{Ca}^{2+}$ , whereupon it emits strong fluorescence. Using this nature of the calcium green-1 acetoxymethyl derivative, the present inventors prepared nanospheres having said derivative adsorbed on the surface. The nanospheres were then encapsulated into fusogenic liposomes and the efficiency of introduction of the nanospheres into the cytoplasm was evaluated. In substantial absence of calcium in the cytoplasm, fluorescence was hardly detectable in each of the groups under test. Even when the intracellular calcium concentration was elevated by the action of calcium ionophore, no fluorescence was detected in the cells on which the nanospheres alone or the nanosphere-encapsulating, non-fusogenic liposomes had been acted. Fluorescence was barely detectable in the cells on which was acted the calcium green-1 acetoxymethyl derivative alone as it was passively transported into the cytoplasm. However, the cells treated with the nanosphere-encapsulating, fusogenic liposomes emitted at least 20 times as much fluorescence as the cells treated solely with the calcium green-1 acetoxymethyl derivative, thus demonstrating the ability of the fusogenic liposomes to permit efficient and positive introduction of nanospheres into the cytoplasm.

These results show that using the fusogenic

liposomes, 20-nm<sup>φ</sup> nanospheres were directly introduced into the cytoplasm with a very high efficiency. The results also show that using the fusogenic liposomes, not only molecules but also vesicles could be introduced into the cytoplasm. It is therefore shown that the fusogenic liposomes can achieve not only spatial dynamic control of substances within cells but also temporal dynamic control such as slow release of drugs from the vesicles introduced into the cell.

10 [Example 1] Fusogenic Liposome Assisted Introduction of  
Rhodamine-PE Encapsulating Polyurea Nanospheres  
and FITC-Dextran Encapsulating Poly(lactic  
acid) Nanospheres into the Cytoplasm

It was found in the Reference Example that using the fusogenic liposomes, even the nanospheres or vesicles having a diameter of 20 nm could be efficiently introduced into the cytoplasm. Based on this finding, the present inventors prepared nanospheres capable of slow release of drugs, attempted to introduce them into the cytoplasm and checked for their stability in the cell. Two models were prepared for the future nanospheres capable of slow release of drugs into the cytoplasm; one was polyurea nanospheres encapsulating rhodamine-PE as a fat-soluble drug model and the other was poly(lactic acid) nanospheres (PLA nanospheres) encapsulating FITC-dextran as a model for a drug in aqueous solution.

These two kinds of nanospheres were encapsulated in fusogenic liposomes. After allowing the liposomes to act

on cells, their stability in the cell was examined by confocal laser microscopy.

#### Materials in Experiment

Diacyl phosphatidylethanolamine-N-lissamine rhodamine  
 5 B sulfonyl (rhodamine-PE) was purchased from Avanti Polar  
 Lipods, INC. Polyvinyl alcohol (PVA), tolylene  
 diisocyanate (TDI), soybean oil, poly(lactic acid) (MW =  
 5000; PLA), Span 80 and methylene chloride were purchased  
 from Wako Pure Chemical Industries, Ltd. FITC-Dextran (MW  
 10 = 150000) was purchased from Sigma. Other reagents were  
 the same or substantially the same as those described in  
 the Reference Example (see under "Materials in  
 Experiment").

#### Methods of Experiment

15 <Preparing rhodamine-PE encapsulating polyurea nanospheres  
 (Ph-polyurea nanospheres)>

The Ph-polyurea nanospheres were prepared by  
 interfacial polymerization as follows. A portion (20 mL)  
 of 0.5% PVA/H<sub>2</sub>O was homogenized at 20000 rpm; a mixture of  
 20 TDI (2.4 g), soyben oil (2.5 g) and rhodamine-PE (0.5  
 mg/500 mL) was added slowly and homogenized for 3 minutes  
 to prepare an oil-in-water (O/W) emulsion. Another portion  
 (100 mL) of 0.5% PVA/H<sub>2</sub>O was homogenized at 10000 rpm and  
 the previously homogenized mixture was added slowly and  
 25 homogenized for an additional 10 minutes to prepare an  
 oil-in-water-in-water [(O/W)/W] emulsion. To reinforce the  
 surfaces of the nanospheres, 100 mL of 0.5% PVA/H<sub>2</sub>O was  
 added and homogenized for 20 minutes, then homogenized at

7000 rpm for 2 hours to prepare Rh-polyurea nanospheres. To purify, the polyurea nanospheres were layered onto a 10%, 20%, 30%, 40% and 50% sucrose step density gradient and centrifuged at 24000 rpm for 2 hours (SW28.1, Beckman).

- 5 The Rh-polyurea nanosphere fraction at the the 30% - 40% sucrose interface was recovered and used in the experiment.  
<Preparing FITC-dextran encapsulating PLA nanospheres (FITC-PLA nanospheres)>

The FITC-PLA nanospheres were prepared by interfacial  
10 precipitation as follows. One gram of PLA and 1 mL of Span 80 were added to 30 mL of  $\text{CH}_3\text{Cl}_2$  and homogenized at 10000 rpm; 5 mL of FITC/dextran (100 mg/mL) was added slowly and homogenized for 1 minute to prepare a water-in-oil (W/O) emulsion; this emulsion was slowly added to 100 mL of 0.5%  
15 PVA/ $\text{H}_2\text{O}$  and homogenized for an additional 5 minutes to prepare a water-in-oil-in-water [(W/O)/W emulsion. The emulsion was further homogenized at 7000 rpm for 1 hour to prepare FITC-PLA nanospheres which were purified by sucrose step density-gradient centrifugation. The FITC-PLA  
20 nanosphere fractions at the 0 - 10% and 10 - 20% sucrose interfaces were recovered and used in the experiment.  
<Preparing and purifying fusogenic liposomes>

Liposomes and fusogenic liposomes were prepared by a modification of the procedures described in the Reference  
25 Example (see under "Preparing and purifying liposomes and fusogenic liposomes" in "Methods of Experiment"). In the preparation of Rh-polyurea nanosphere encapsulating fusogenic liposomes, the method of Bangham et al. was

repeated, with the fluorescence intensity of the nanospheres being adjusted to 50000 (Ex, 550 nm; Em, 590 nm), to prepare liposomes. Thereafter, the liposome solution was layered onto a 10%, 20%, 30%, 40% and 50% sucrose density gradient and centrifuged at 24000 rpm for 2 hours (SW28.1, Beckman); the fraction at the 20 - 30% sucrose interface was recovered and the liposomes were purified. The purified liposomes were reacted with Sendai virus and the reaction product was layered onto a 20%, 30%, 35%, 45% and 50% sucrose density gradient and centrifuged at 24000 rpm for 2 hours (SW28.1, Beckman); the fractions at the 30 - 35% and 35% - 40% sucrose interfaces were recovered to give a purified form of Rh-polyurea nanosphere encapsulating fusogenic liposomes.

In the preparation of FITC-PLA nanosphere encapsulating fusogenic liposomes, liposomes were first prepared, with the fluorescence intensity of the nanospheres being adjusted to 10000 (Ex, 490 nm; Em, 529 nm). The liposomes were reacted with Sendai virus and the reaction product was layered onto a 10%, 20%, 30%, 40% and 50% sucrose density gradient and centrifuged at 24000 rpm for 2 hours (SW28.1, Beckman). The fusogenic liposomes prepared from the nanospheres recovered from the fractions at the 0 - 10% and 10 - 20% sucrose interfaces were present at the 10 - 20% and 20% - 30% sucrose interfaces, respectively; these fractions were recovered to give a purified form of FITC-PLA nanosphere encapsulating fusogenic liposomes.

<Introducing nanospheres into cultured cells>

4 x 10<sup>4</sup> Ltk<sup>-</sup> cells were seeded on 4-well chamber slides. After one day, the cells were washed with PBS(-) and exposed for 1 hour to the action of the Rh-polyurea nanospheres, Rh-polyurea nanosphere encapsulating fusogenic liposomes (fluorescence intensity, 10000; Ex, 550 nm; Em, 590 nm), FITC-PLA nanospheres, and FITC-PLA nanosphere encapsulating fusogenic liposomes (fluorescence intensity, 700; Ex, 490 nm; Em, 520 nm). After washing with PBS(-), the cells were cultured in an Eagle's MEM medium. After one day, the medium was replaced by an anti-stain agent, or a 0.1% p-phenylenediamine/Eagle's MEM medium and the cells were examined.

<Examination by confocal laser microscopy>

Examination was made in accordance with the procedure described in the Reference Example (see under "Examination by confocal laser microscopy" in "Methods of Experiment"). For rhodamine-PE, the conditions were Ex of 568 nm and Em of 585 nm (585 LP); for FITC-dextran, the conditions were Ex of 488 nm and Em of 540 nm (540 DF).

The prepared Rh-polyurea nanospheres and FITC-PLA nanospheres, as well as the fusogenic liposomes encapsulating the RH-polyurea nanospheres and the fusogenic liposomes encapsulating the FITC-PLA nanospheres were examined by confocal laser microscopy; without doubt, the two kinds of nanospheres were prepared.

The fluorescence of the substances encapsulated in the two kinds of nanospheres occurred in the positions on

transmission micrographs where the fusogenic liposomes were observed. Thus, it became clear that the polyurea nanospheres and PLA nanospheres could be encapsulated into fusogenic liposomes. According to the transmission  
5 photographs, the polyurea nanospheres prepared in Example 1 had particle sizes of about 200 nm and the PLA nanospheres had particle sizes of about 500 nm. These nanospheres were encapsulated in the fusogenic liposomes, indicating that even fairly large nanospheres could be encapsulated in the  
10 fusogenic liposomes. Since the polyurea nanospheres could be encapsulated in the fusogenic liposomes, it became clear that according to the invention, one could produce fusogenic liposomes that encapsulated a large volume of hydrophobic drugs which had been encapsulated with only low  
15 efficiency by the conventional methods of liposome preparation. An examination made one day after the nanosphere encapsulating fusogenic liposomes were allowed to act on the Ltk<sup>-</sup> cells offered positive evidence for the presence of the nanospheres within the cells. The Ltk<sup>-</sup>  
20 cells into which the nanospheres were introduced by means of the fusogenic liposomes showed no morphological changes after the lapse of one day and this indicates the absence of superficial cell injury.

These results show that the fusogenic liposomes can  
25 introduce 100 - 500 nm nanospheres into the cytoplasm and that the introduced nanospheres remain stable in the cytoplasm even after the passage of a day. Based on this finding, the inventors studied the release of drugs from



the nanospheres in Example 2.

[Example 2] Slow Drug Release from FITC-Dextran  
Encapsulating

### Poly(lactic acid) Nanospheres

5        It was found in Example 1 that the prepared  
nanospheres could be introduced into the cytoplasm by means  
of the fusogenic liposomes and that they remained stable  
within the cell for at least 24 hours. However, if these  
nanospheres are incapable of releasing drugs at slow rate,  
10    it is of course impossible to have drugs released slowly in  
cells. The present inventors therefore chose FITC-dextran  
as a model drug and conducted an experiment to evaluate its  
slow releasabiity from the prepared FITC-PLA nanospheres.

### Materials in Experiment

15        The reagents were the same or substantially the same  
as those described in Example 1 (see under "Materials in  
Experiment").

### Methods of Experiment

<Evaluating the slow release of drug from FITC-dextran  
20    encapsulating PLA nanospheres>

The FITC-PLA nanospheres were suspended in BSS(-) at  
a fluorescence intensity of 100000 (Ex, 490 nm; Em, 520 nm)  
and incubated at 37°C. At days 0, 1, 2, 3 and 4,  
centrifuge was conducted at 10000 rpm to remove the  
25    nanospheres and the amount of FITC-dextran in the  
supernatant was measured with a fluorescence photometer at  
Ex of 490 nm and Em of 520 nm.

### Results and Discussion

The release of FITC-dextran from the FITC-PLA nanospheres prepared in Example 1 was observed. As Fig. 1 shows, the FITC-dextran was released in only about 6% even at day 4 and its slow release was evident. The PLA nanospheres or microspheres are generally said to release drugs over a period from about 3 weeks to three months and in one report, the release was ten-odd percent at day 3. Compared to the data reported previously, the PLA nanospheres prepared in Example 1 allow for somewhat slower drug release. However, the rate of drug release from the PLA nanospheres can be controlled by adjusting the molecular weight of PLA and the size of spheres, so the slow release characteristics of drugs in the cytoplasm can be controlled by appropriately changing the conditions of nanosphere preparation.

Considering the result of Example 2 in combination with that of Example 1, it is concluded that the nanospheres allowed the drug to be slowly released in the cytoplasm.

## 20 [Example 3] Studies on the Stabilization and Controlled Release of Plasmid DNA Encapsulated in Gelatin Nanospheres

The T7 expression system enables efficient gene expression if bacteriophage T7 RNA polymerase and plasmid DNA having the T7 promoter sequence are both present in the cytoplasm. However, due to the low stability of plasmid DNA in the cytoplasm, gene is rapidly decomposed away, leading to only a very brief period of gene expression.

Therefore, in order for the T7 expression system to express genes in therapeutically necessary amount for the necessary period, the genes in the cytoplasm must be controlled dynamically by stabilizing the plasmid DNA in the cytoplasm and allowing it to be released slowly. Nanospheres are used as formulations capable of slow release of drugs and as shown in the Reference Examples and Examples 1 and 2, they can not only be introduced into but can also be released slowly in the cytoplasm. While nanospheres can be made of various materials including PLA mentioned in Example 1, gelatin has particularly good biocompatibility and can easily form gelatin nanospheres by coacervation with plasmid DNA and other high-molecular weight substances. For experimental purposes, gelatin nanospheres are considered to provide ease in evaluating the slow intracellular release of drugs using gene expression as a marker because the release pattern can be conveniently controlled by crosslinking and because the gelatin nanospheres permit faster drug release than the PLA nanospheres which are generally held to be capable of fast enough drug release. With these points taken into consideration, the gelatin nanospheres are believed to be most suitable for use at the first stage of applying the slow intracellular release to the T7 expression system. Therefore, in Example 3, gelatin nanospheres containing plasmid DNA were prepared by phase separation and studies were made on their ability to stabilize plasmid DNA, as well as the control of its release.

### Materials in Experiment

Gelatin (type A, produced from porcine skin) was purchased from Sigma. Other reagents, 2-morpholinoethanesulfonic acid monohydrate (MES), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, WSC), Na<sub>2</sub>SO<sub>4</sub> and glycine were purchased from Wako Pure Chemical Industries, Ltd. Pentobarbital was purchased from Dainippon Pharmaceutical Co., Ltd. The other reagents were the same or substantially the same as those described in the Reference Example (see under "Materials in Experiment").

### Methods of Experiment

<Preparing pT7- $\beta$ -globin-IRES-L-polyA encapsulating gelatin nanospheres>

The pT7- $\beta$ -globin-IRES-L-polyA encapsulating gelatin nanospheres were prepared by phase separation. A hundred milliliters of 5% gelatin solution and a 45 mM Na<sub>2</sub>SO<sub>4</sub> solution containing pT7- $\beta$ -globin-IRES-L-polyA(0.2 mg/mL) were mixed in a 1.5 mL Eppendorf tube and incubated in a water bath at 57°C for 10 minutes. Thereafter, the mixture was vortexed for 1 minute, layered on top of a 35%, 55% and 66% sucrose density gradient and centrifuged at 19000 rpm and 20°C for 1 hour (SW55, Beckman). A 55% sucrose layer containing the pT7- $\beta$ -globin-IRES-L-polyA encapsulating gelatin nanospheres was recovered and subjected to the experiment.

<Crosslinking the gelatin nanospheres>

After preparing the gelatin nanospheres, the 55%

sucrose layer was diluted 2-fold and reacted with an MES  
buffer solution (0.2 M MES, 0.1 or 0.5 mg/mL of EDC, pH  
4.5) that was added in a 1/10 volume. Thirty minutes  
later, glycine was added to give 0.2 M, thereby quenching  
5 the reaction.

<Preparing fraction S-9>

Wistar rats were fasted overnight, anesthetized with  
peritoneally administered pentobarbital and had the blood  
taken from the heart until death. Ice-cooled 0.15 M KCl  
10 was perfused through the portal vein into the liver in  
order to remove a maximum volume of the hepatic blood. The  
liver was extracted and washed with ice-cooled 0.15 M KCl;  
after adding ice-cooled 0.15 M KCl in an amount three times  
its weight, the liver was homogenized under cooling with  
15 ice. The homogenate was centrifuged at 9000g and 4°C for  
10 minutes. The supernatant was used as fraction S-9.  
This fraction was quick frozen with liquid nitrogen and  
stored at -80°C.

<Studying the stabilization of plasmid DNA>

20 The pT7- $\beta$ -globin-IRES-L-polyA encapsulating gelatin  
nanospheres were put into a 5% solution of fraction S-9 and  
reaction was performed at 37°C. After a specified time,  
the S-9 solution was removed by centrifuging and the  
remaining nanospheres were destroyed by treatment with  
25 trypsin; the plasmid DNA was stained with ethidium bromide  
and its stability was evaluated by agarose gel  
electrophoresis.

<Release of plasmid DNA from gelatin nanospheres>

The pT7- $\beta$ -globin-IRES-L-polyA encapsulating gelatin nanospheres were crosslinked at various concentrations by the method described above and treated in 1.25 mg/mL of trypsin for a specified time. Thereafter, the nanospheres  
5 were removed by centrifuging and the amount of plasmid DNA released was determined with DABA.

### Results and Discussion

In order to evaluate the stability of plasmid DNA in cells, the degradability of plasmid DNA was examined by  
10 agarose gel electrophoresis using fraction S-9 of the rat liver homogenate (Fig. 2). When the naked plasmid DNA was reacted with fraction S-9, it was substantially degraded within 30 minutes; on the other hand, when it was encapsulated within the gelatin nanospheres, the plasmid  
15 DNA remained stable at 60 minutes and thereafter. This showed that encapsulating plasmid DNA within nanospheres was effective in increasing its stability. According to the release pattern of plasmid DNA from the gelatin nanospheres in trypsin solution after treatment with  
20 various concentrations of the crosslinker, the release of plasmid DNA was suppressed by increasing the concentration of the crosslinker (Fig. 3).

In the T7 expression system which expresses genes in the cytoplasm where plasmid DNA tends to degrade faster  
25 than in the nucleus, the stability of the plasmid DNA in nanospheres and the rate of its release from them are two key factors in adjusting the amount and period of gene expression. The results of Example 3 suggest that the

stability of plasmid DNA is improved by encapsulating them within gelatin nanospheres and that the rate of release of a drug (which is plasmid DNA in the experiment described above) can be controlled by altering the conditions of nanosphere preparation. This is evidence for the possibility of adjusting the amount and period of gene expression.

[Example 4] Gene Expression by Plasmid DNA Encapsulated in Gelatin Nanospheres Encapsulated in Fusogenic Liposomes

In Example 3, it was shown that the intracellular stability of plasmid DNA was improved by encapsulating it in nanospheres and that the amount of release of plasmid DNA could be controlled by treating gelatin nanospheres with a crosslinker. Based on these results, the present inventors attempted to increase the period and amount of gene expression by the T7 expression system. To this end, in Example 4, gelatin nanospheres encapsulating PT7- $\beta$ -globin-IRES-L-polyA which was a luciferase expressing plasmid DNA having the T7 promoter were encapsulated in fusogenic liposomes and introduced into T7 RNA polymerase producing cells to see how the plasmid DNA slowly released in the cell would affect the pattern of gene expression by the T7 expression system.

Materials in Experiment

As a luciferase assay system, PicaGene, a product of TOYO INK MFG. CO., LTD. was used. The other reagents were the same or substantially the same as those described in



the Reference Example and Examples 1 - 3 (see under "Materials in Experiment").

#### Methods of Experiment

##### <Preparing and purifying fusogenic liposomes>

5 Fusogenic liposomes were prepared by a partial modification of the procedures described in the Reference Example (see under "Preparing and purifying liposomes and fusogenic liposomes"). To be specific, Sendai virus whose RNA was fragmented by illumination with uv light (2000  
10 J/m<sup>2</sup>) were reacted with liposomes to obtain fusogenic liposomes.

##### <Cultured cells>

Monkey kidney epithelial cells LLC-MK2#10 were cultured in an Eagle's MEM medium containing 10% fetal calf  
15 serum (FCS).

##### <Introducing a substance into cultured cells>

5 x 10<sup>4</sup> LLC-MK2#10 cells were seeded on 12-well plates. After one day, the cells were washed with PBS(-). In a separate step, pT7-β-globin-IRES-L-polyA encapsulating  
20 fusogenic liposomes, pT7-β-globin-IRES-L-polyA encapsulating gelatin nanosphere encapsulating fusogenic liposomes and pT7-β-globin-IRES-L-polyA encapsulating gelatin nanospheres were diluted in a serum-free Eagle's MEM medium at an appropriate concentration to incorporate  
25 the same amount of genes. Such fusogenic liposomes or nanospheres were allowed to act on the washed cells for 1 hour. After washing with PBS(-), the cells were cultured in an Eagle's MEM medium.

<Measuring the luciferase activity>

Luciferase activity was measured with the luciferase assay system and a luminometer (Lumit LB 9507, Berthold). The activity was expressed in terms of relative light  
5 unit/well.

For other methods of experiment, see "Methods of Experiment" in Example 3.

Results and Discussion

The plasmid DNA containing nanospheres crosslinked  
10 with 0.01 mg/mL of EDC were encapsulated in the fusogenic liposomes, introduced into T7 RNA polymerase producing cells and checked for their effectiveness using the daily profile of gene expression as a marker (Fig. 4). The fusogenic liposomes encapsulating only plasmid DNA and the  
15 plasmid DNA encapsulating nanospheres, both having been processed to have the same amount of genes, were similarly allowed to act on the cells and the daily profile of luciferase activity was measured. The result was substantially nil. In the group treated with the fusogenic  
20 liposomes encapsulating the plasmid DNA encapsulating nanospheres, very high luciferase activity was observed at day 1 and day 2 after gene introduction. The efficiency of gene expression was substantially the same at day 1 and day 2 after gene introduction. The pattern of gene expression  
25 in the T7 expression system has been shown to be such that gene expression disappears significantly at day 1 of gene introduction, so the result of Example 4 seems to suggest that a certain extension of the period of gene expression

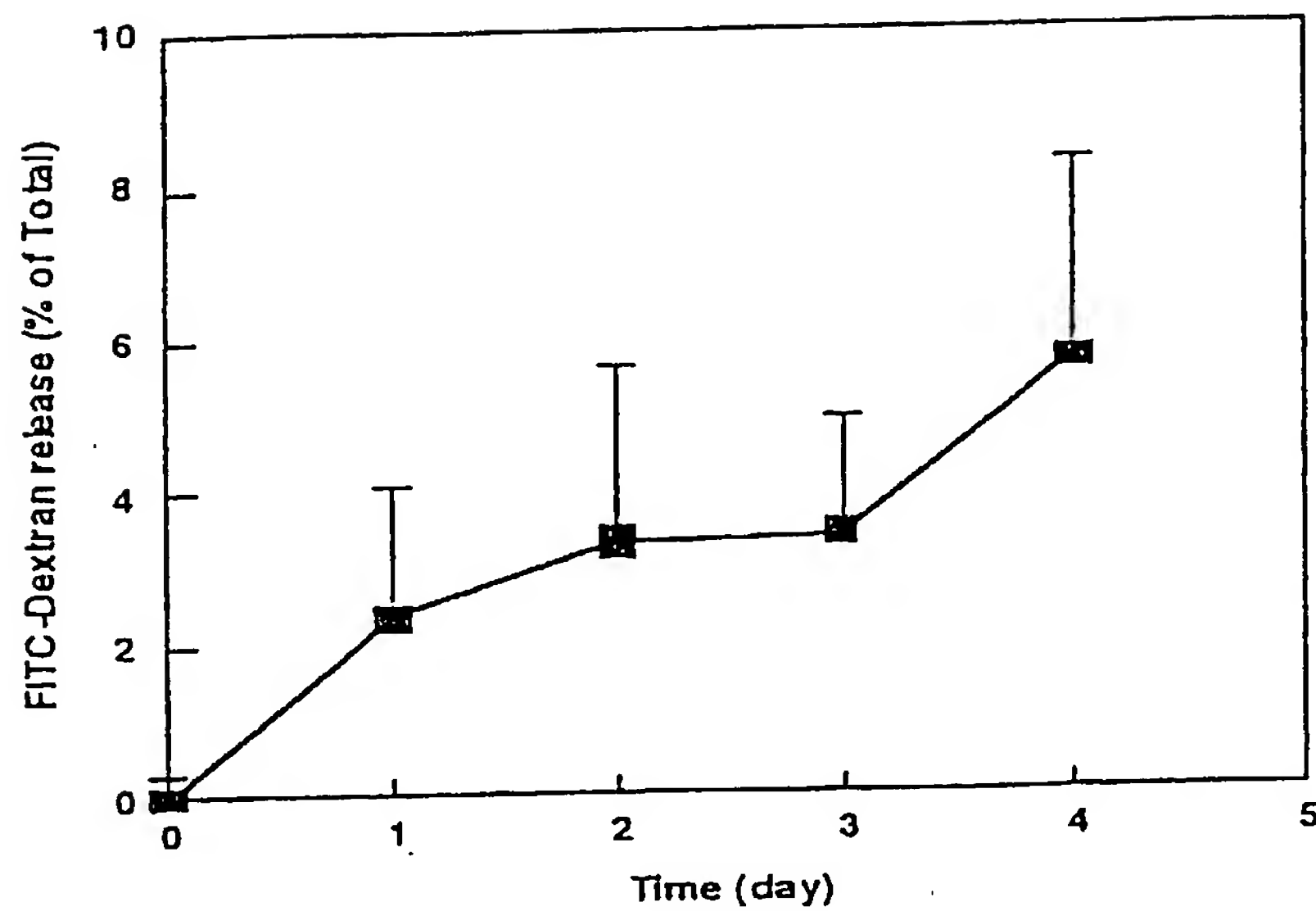
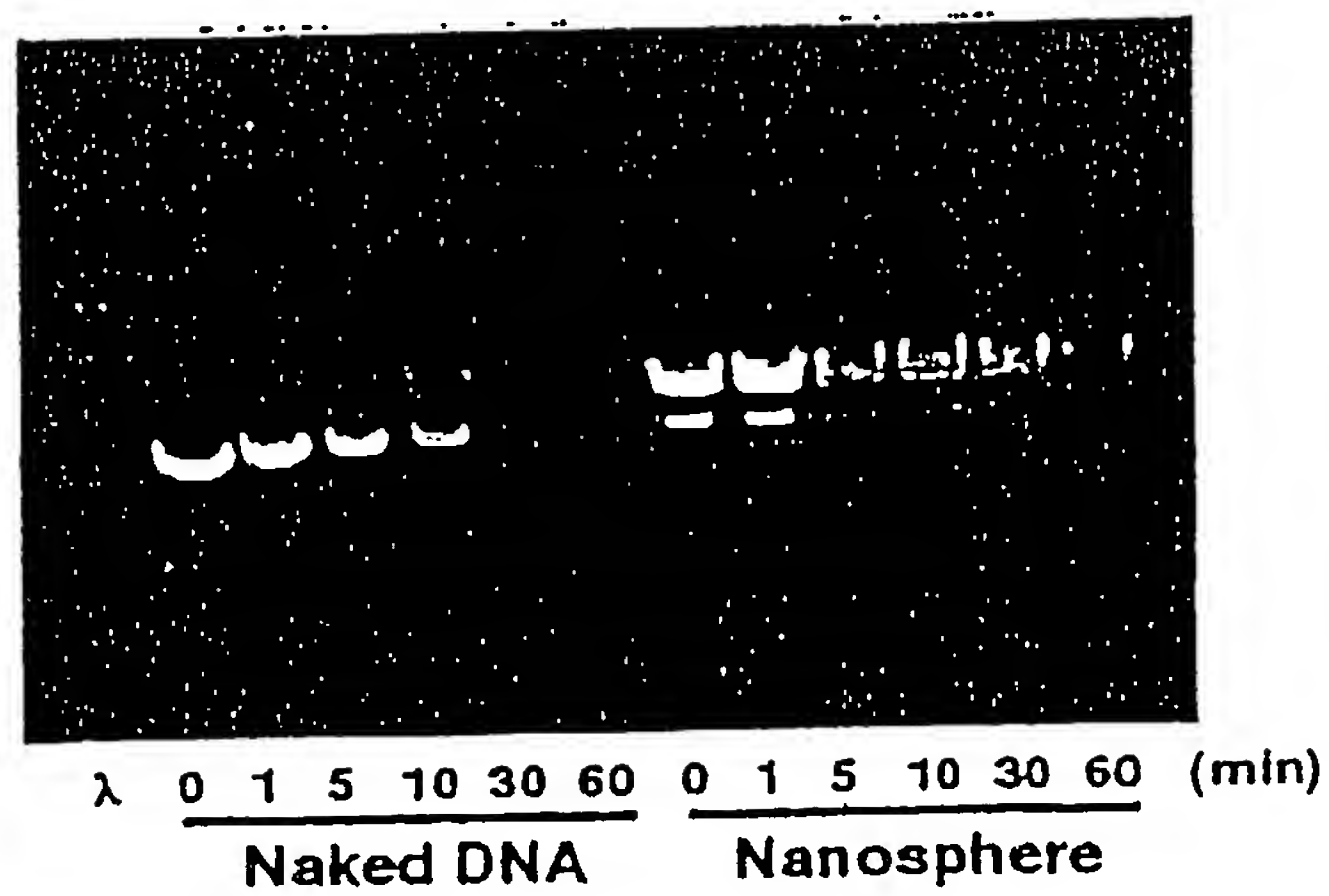
could be achieved by incorporating a gene in nanospheres and introducing it into the cytoplasm. Under the conditions used in Example 4, the gene expression dropped at day 4. However, in Example 4, the efficiency of gene expression increased and the expressed gene remained stable for at least 2 days. Hopefully, the amount and period of gene expression can be adjusted by altering the conditions of nanosphere preparation.

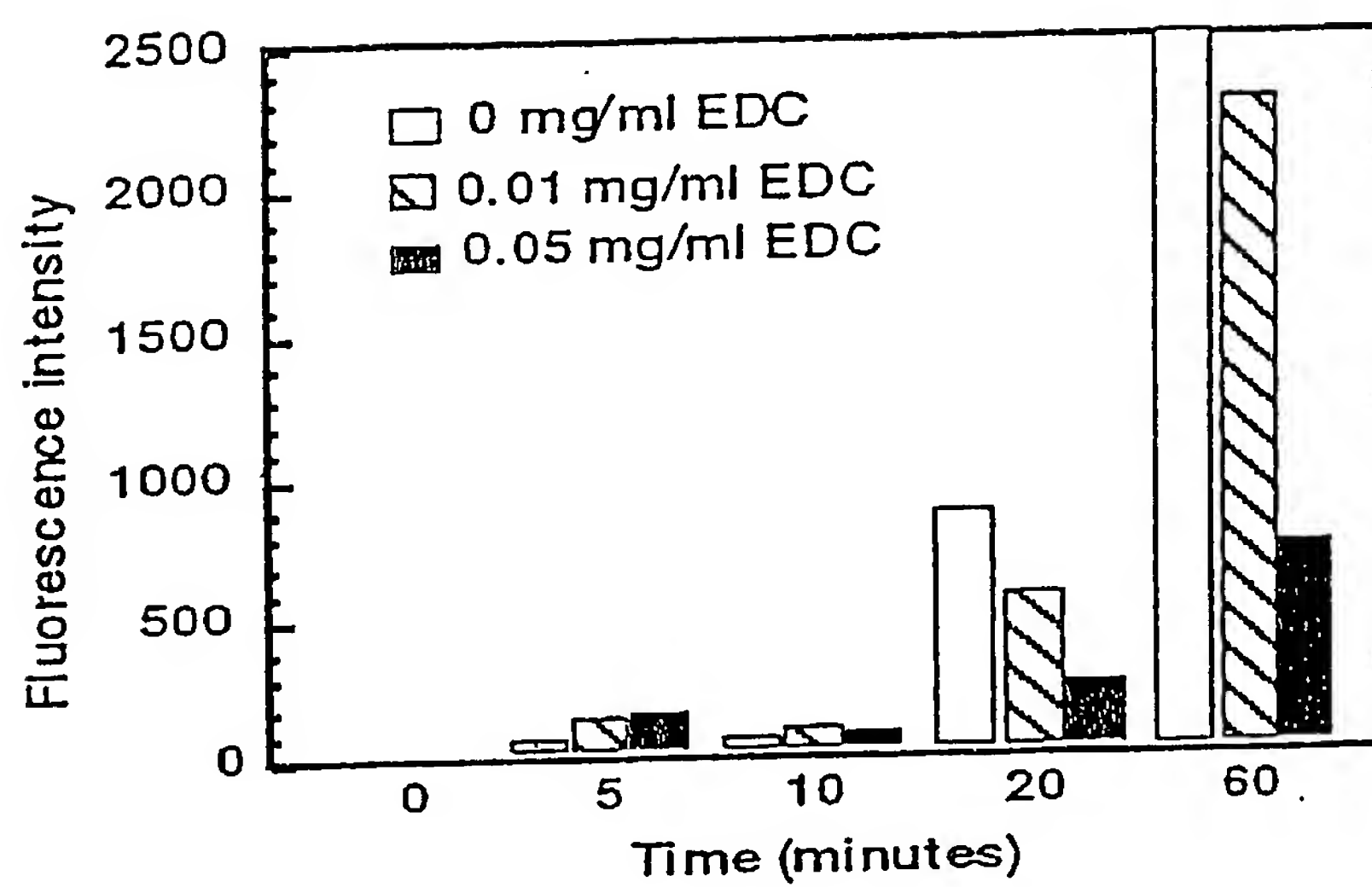
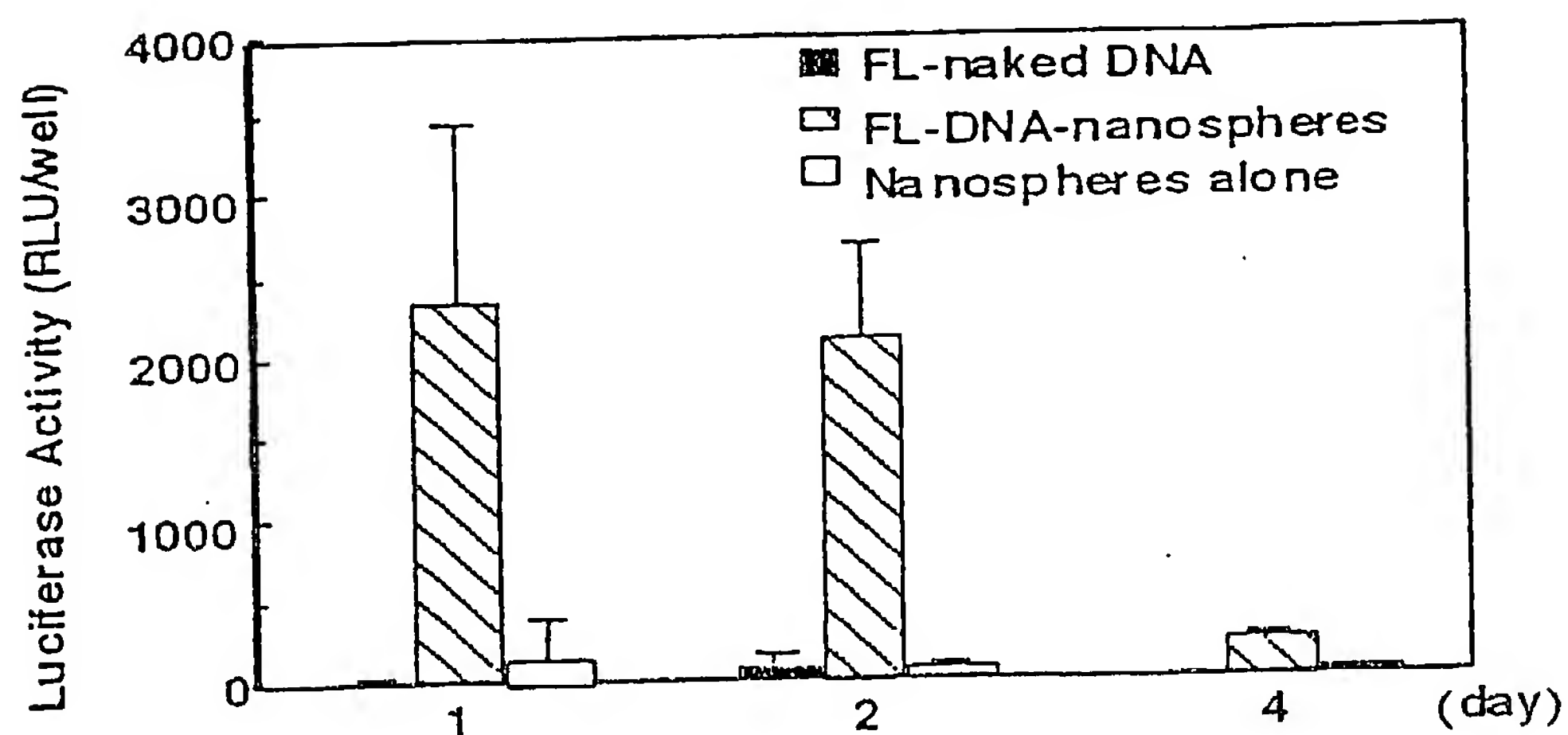
# CLAIMS

1. A slow-release composition for introducing a physiologically active substance into the cytoplasm, comprising nanospheres that encapsulate the physiologically active substance and which are encapsulated in liposomes having fusogenic capability.
2. The composition according to claim 1, wherein the nanospheres have a particle size of 10 - 600 nm.
3. The composition according to claim 1 or 2, wherein the fusogenic capability derives from Sendai virus.
4. The composition according to any one of claims 1 - 3, wherein the physiologically active substance is selected from the group consisting of low-molecular weight drugs, proteins and genes.
5. A process for producing a composition that contains a physiologically active substance and which allows it to be slowly released in the cytoplasm, comprising the steps of encapsulating a physiologically active substance in nanospheres and encapsulating said nanospheres in liposomes having fusogenic capability.
6. A method of introducing a physiologically active substance into an animal by contacting a viable cell with the composition according to any one of claims 1 - 4.

# ABSTRACT

A substance of interest is contained in nanospheres which are then encapsulated in fusogenic liposomes to  
 5 prepare transport carriers that allow physiologically active substances, especially those having high molecular weight such as proteins and genes, to be introduced into cells efficiently and which permit the introduced active substance to be released in the cell at controlled rate.  
 10 The fusogenic liposomes are prepared by conferring the fusogenic capability of Sendai virus to known liposomes.

*Fig. 1**Fig. 2*

*Fig. 3**Fig. 4*



Page 1 of 2 Pages

[X] Original [ ] Supplemental

Atty. Docket

**Combined Declaration for Patent Application and Power of Attorney**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD AND FORMULATION FOR SUSTAINED INTRACELLULAR DRUG RELEASE

the specification of which (check one)

☐ is attached hereto;☐ was filed in the United States under 35 U.S.C. §111 on \_\_\_\_\_, as U.S. Appln. No. \_\_\_\_\_\*; or

☒ was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/JP00/01267 filed Mar. 3, 2000, entry requested on \_\_\_\_\_\*; national stage application received U.S. Appln. No. \_\_\_\_\_\*; §371/§102(e) date \_\_\_\_\_\* (\* if known)

and was amended on \_\_\_\_\_ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 and 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed:

<u>249845/1999</u>	<u>Japan</u>	<u>3/9/1999</u>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO
<u>                    </u>	<u>                    </u>	<u>                    </u>	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day Month Year Filed)	YES	NO

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or prior PCT application(s) designating the U.S. listed below, or under §119(e) of any prior U.S. provisional applications listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information as defined in 37 C.F.R. §1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

<u>                    </u>	<u>                    </u>	<u>                    </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
<u>                    </u>	<u>                    </u>	<u>                    </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
<u>                    </u>	<u>                    </u>	<u>                    </u>
(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)



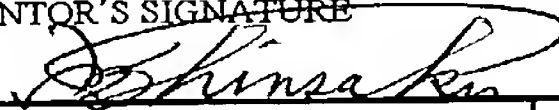


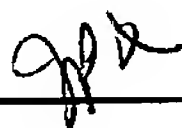


As a named inventor, I hereby appoint the following registered practioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

**All of the practioners associated with Customer Number 001444**

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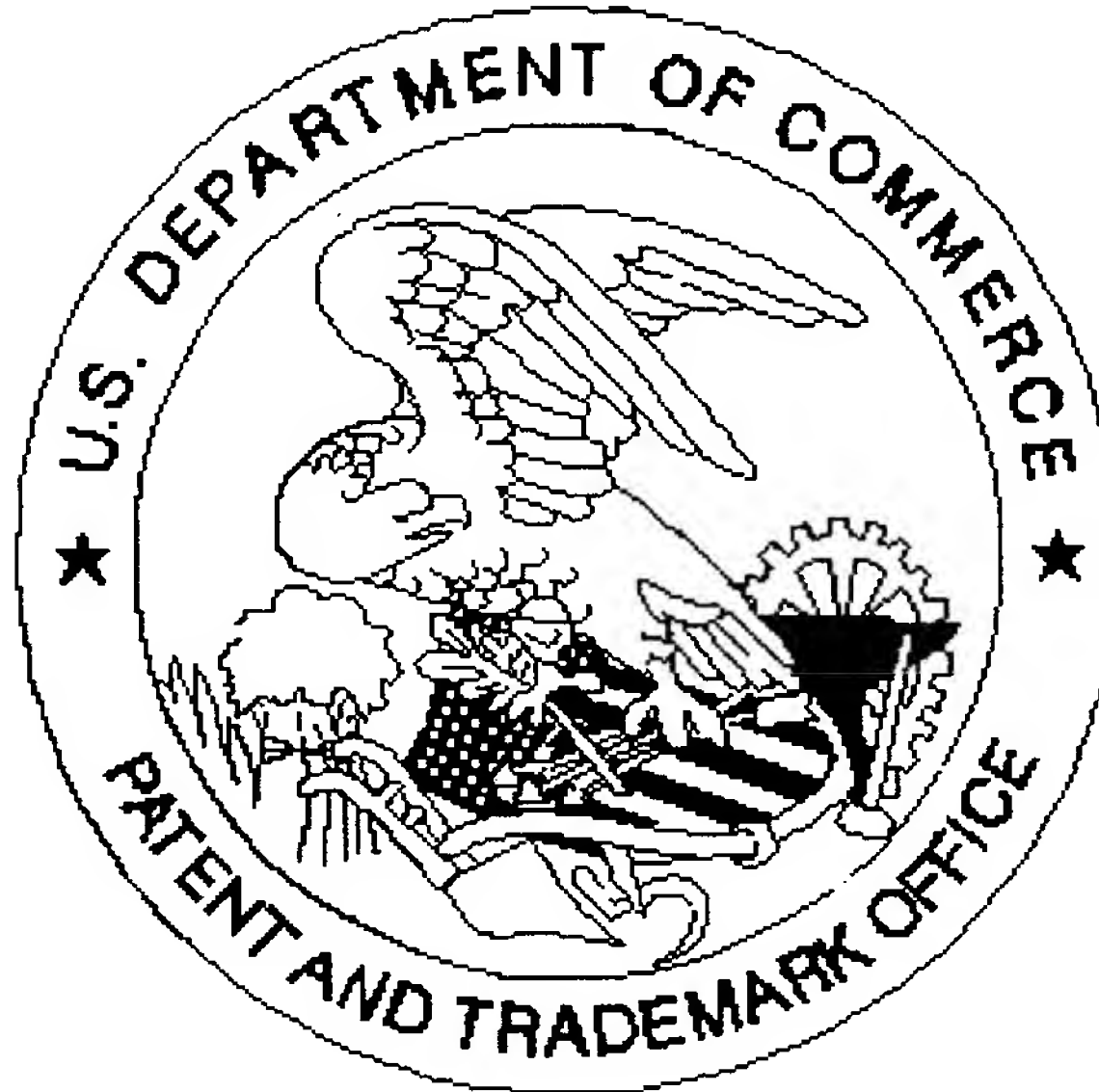
The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from YUASA AND HARA as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

U.S. Application filed \_\_\_\_\_, Serial No. \_\_\_\_\_  
PCT Application filed March 3, 2000, Serial No. PCT/JP00/01267

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ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

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